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Synergistic Interaction between Silver Nanoparticles and Membrane-Permeabilizing Antimicrobial Peptides▽

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Silver nanoparticles, as well as antimicrobial peptides (AMPs), can be used to fight infectious diseases. Since AMPs are known to permeabilize bacterial membranes and might therefore help silver nanoparticles to access internal target sites, we investigated their combined activities and showed synergistic effects between polymyxin B and silver nanoparticles for gram-negative bacteria.

The worldwide escalation of bacterial resistance to conventional medical antibiotics is a serious concern for modern medicine (4). In view of this development, the aim of our study was to investigate the antimicrobial and hemolytic properties of silver(I) ions and silver nanoparticles in combination with different antimicrobial peptides (AMPs). The synergistic action of antimicrobial agents can reduce the need for high dosages and minimize side effects (5, 9, 18, 19). Examples include PGLa plus magainin 2 from *Xenopus laevis* (18) and β-lactam amoxicillin with silver nanoparticles (9), but a combination of silver nanoparticles or silver ions with AMPs has not been tested. We therefore examined commercial polymyxin B, gramicidin A and alamethicin (from Sigma-Aldrich, Buchs, Switzerland), gramicidin S extracted from *Aneurinibacillus migulanus* (2), and PGLa and magainin 2, which was synthesized in our laboratory. All noncommercial peptides were purified on a reverse-phase-high-performance liquid chromatography system to greater than 94%.

The cyclic polycationic lipopeptide polymyxin B is effective against gram-negative bacteria, interacting with lipid A and disrupting their outer membranes (16). Gramicidin A and alamethicin are hydrophobic and act against gram-positive bacteria by forming ion-selective channels (3) or barrel-stave pores (11), respectively. Gramicidin S is a cyclic β-sheet peptide (8), while PGLa and magainin 2 are α-helical (12). They are all cationic and amphipathic and have similar activities against gram-negative and gram-positive bacteria, forming transmembrane pores (12, 14). In combination with these diverse AMPs, we tested silver nitrate (VWR, Darmstadt, Germany) and silver nanoparticles (mean diameter, 25 nm) stabilized by a nonbiocidal carbon matrix (data not shown) (NovaCentrix, Austin, TX). Electron microscopy demonstrated that these nanoparticles accumulate at both the outer and inner membranes of bacteria (13). The exact mode of action of silver ions and silver nanoparticles is unknown, but they seem to significantly reduce cellular chemiosmotic potential (6, 10). Furthermore, it has been reported that within a period of 24 h, less than 5 μM of free silver(I) ions are released into solution (13). Therefore, the antimicrobial effect of silver nanoparticles cannot be attributed to the release of silver(I) ions from the nanoparticles, yet sensitivity to silver may differ for various bacterial strains or when different media are used (15).

TABLE 1. MIC values for silver and peptides[a]

Strain[b]	MIC for $AgNO_3$ (μg/ml)	MIC for AgNP (μg/ml)	MIC for PGLa (μg/ml)	MIC for Mag2 (μg/ml)	MIC for GS (μg/ml)	MIC for PMB (μg/ml)	MIC for Alam (μg/ml)	MIC for GA (μg/ml)
E. coli (DSM 498)	8–16	64	8–16	16–32	8	1–2	256	>128
A. calcoaceticus (DSM 586)	8–16	RS	8–16	16–32	16	1	256	>128
E. helveticus (DSM 18396)	64–128	64	8–16	8–16	4–8	1–2	256	>128
A. bestiarum (DSM 13956)	1–2	8–16	>256	>256	32	16	256	>128
P. myxofaciens (DSM 4482)	1–2	8–16	>256	>256	16–32	16–32	256	>128
P. fluorescens (DSM 50090)	1–2	8–16	>256	>256	16	128	128–256	>128
B. subtilis (DSM 347)	8–16	32–64	8–16	16–32	2–4	8–16	8	1–2
K. rhizophila (DSM 348)	8	4–8	8–16	16–32	4–8	16	8	1–2
M. luteus (DSM 1790)	8–16	4–8	4–8	16–32	2–4	4–8	8	1–2

[a] $AgNO_3$, silver nitrate; AgNP, silver nanoparticles; PGLa, GMASKAGAIAGKIAKVAL-KAL-NH_2; Mag2, magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS); GS, gramicidin S (*cyclo*[VOL^{D}FP]$_2$, where D shows the stereocenter of the amino acid); PMB, polymyxin B {(S)-6-methyloctanoyl-BTB-*cyclo*[BB^{D}FLBBT]; B, diaminobutyric acid}; Alam, alamethicin (Ac-XPXAXAQXVXGL-XPVXXEQ-Fol; X, α-aminoisobutyric acid); GA, gramicidin A (HCO-VGA^{D}LA^{D}VVV^{D}W^{D}LW^{D}LW^{D}L-NHCH$_2$CH$_2$OH).

[b] *E. coli*, *Escherichia coli; A. calcoaceticus*, *Acinetobacter calcoaceticus; E. helveticus*, *Enterobacter helveticus*; *A. bestiarum*, *Aeromonas bestiarum; P. myxofaciens*, *Proteus myxofaciens; P. fluorescens*, *Pseudomonas fluorescens; B. subtilis*, *Bacillus subtilis; K. rhizophila*, *Kocuria rhizophila; M. luteus*, *Micrococcus luteus*.

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The MIC is the lowest concentration of a (single) antibiotic that inhibits the visible growth of microorganisms (1). Assays were carried out in salt-free Luria/Miller-bouillon growth medium for three different gram-positive and six gram-negative bacterial strains. To dissolve silver nanoparticles for the MIC and hemolytic assays, an 8 mM Tris/HCl buffer [pH 7.6] containing 150 mM NaCl was used. Typical MICs, summarized in Table 1, were around 16 μg/ml of PGLa (for both gram-positive and gram-negative bacteria) and 1 μg/ml of gramicidin A (for gram-positive bacteria only) and polymyxin B (for gram-negative bacteria only). Similar MICs of about 16 μg/ml of both silver nitrate and silver nanoparticles were obtained. It is remarkable that the metallic silver nanoparticles and the ionic solution exhibit similar activities at almost the same mass per volume, given that each nanoparticle contains about 650,000 silver atoms. This observation supports the suggestion that the toxicity of the nanoparticles cannot be attributed to the release of free silver ions (see above) (13).

To detect any synergism between the peptides and silver, a two-dimensional microdilution assay was used (7). Assays were carried out in salt-free Luria/Miller-bouillon growth medium. The combined antibiotic effect of agents A and B (where A is either $AgNO_3$ or silver nanoparticles, and B is one of six AMPs) was calculated as follows: the fractional inhibitory concentration (FIC) index = MIC (A in combination with B)/MIC (A alone) + MIC (B in combination with A)/MIC (B alone). FIC index values above 2.0 indicate antagonistic effects, values between 0.5 and 2.0 indicate additive effects, and values lower than 0.5 indicate synergistic effects (7). The synergistic pair of PGLa and magainin 2 was used as a positive control. The FIC indices in Table 2 show the following results: polymyxin B acts synergistically with silver nanoparticles against all gram-negative bacteria, while with $AgNO_3$ it shows synergy only against *Escherichia coli*, *Enterobacter helveticus*, and *Proteus myxofaciens*. In addition, gramicidin S shows synergy with silver nanoparticles against *E. helveticus*, *P. myxofaciens*, and *Pseudomonas fluorescens*, and with $AgNO_3$ only against *E. helveticus*. All the other combinations work only in an additive manner. '*Acinetobacter calcoaceticus*' was not sensitive to silver nanoparticles even at the highest concentration (1,024 μg/ml). We may nevertheless conclude that silver nanoparticles generally show more synergy than silver(I) ion against most strains.

Hemolytic activity was examined using a modified serial dilution assay carried out in 8 mM Tris/HCl buffer [pH 7.6] containing 150 mM NaCl (17). The ability of AMPs to release hemoglobin from human erythrocytes was measured photometrically at different peptide concentrations. Triton X-100 (Roth, Karlsruhe, Germany) was used to define 100% hemolysis. For every experimental run, the value for autohemolysis was measured and subsequently subtracted from all hemolytic values. All peptides were tested alone and in combination with two different concentrations of $AgNO_3$ and silver nanoparticles to obtain the AMP concentration (in μg/ml) required to induce 50% hemolysis (HC_{50}). It was shown that increasing the concentration of $AgNO_3$ in combination with any AMP enhances its hemolytic activity (Table 3). However, the combination of AMPs with silver nanoparticles does not increase hemolysis. In fact, the HC_{50} value for the silver nanoparticles on their own could not be determined, as they had low levels of

TABLE 2. Synergy among silver and peptides as presented in FIC index[a]

Strain[b]	Synergy control[c] (PGLa/Mag2)	FIC index value for combination of indicated peptide with silver nitrate[c]:						FIC index value for combination of indicated peptide with silver nanoparticles[c]:					
		PGLa	Mag2	GS	PMB	Alam	GA	PGLa	Mag2	GS	PMB	Alam	GA
E. coli (DSM 498)	**0.28 (0.03)**	0.67 (0.06)	1.00 (0.35)	0.83 (0.12)	**0.38 (0.1)**	0.50 (0)	ND	1.00 (0.4)	0.86 (0.46)	0.56 (0.05)	**0.38 (0)**	0.50 (0)	ND
A. calcoaceticus (DSM 586)	**0.26 (0.03)**	1.08 (0.31)	0.79 (0.16)	0.77 (0.18)	0.83 (0.12)	ND	ND	ND	ND	ND	ND	ND	ND
E. helveticus (DSM 18396)	**0.20 (0.04)**	0.71 (0.06)	0.71 (0.06)	**0.32 (0.04)**	**0.29 (0.02)**	ND	ND	0.57 (0.04)	0.59 (0.04)	**0.44 (0.09)**	**0.38 (0.1)**	ND	ND
A. bestiarum (DSM 13956)	**0.28 (0.1)**	ND	ND	0.53 (0.02)	0.50 (0)	ND	ND	ND	ND	0.63 (0.1)	**0.39 (0.09)**	ND	ND
P. myxofaciens (DSM 4482)	**0.29 (0.06)**	ND	ND	0.63 (0.27)	**0.23 (0.1)**	ND	ND	ND	ND	**0.38 (0.1)**	**0.23 (0.03)**	ND	ND
P. fluorescens (DSM 50090)	**0.27 (0.06)**	ND	ND	0.67 (0.11)	0.56 (0.05)	1.00 (0)	ND	ND	ND	**0.48 (0.13)**	**0.27 (0.08)**	1.00 (0)	ND
B. subtilis (DSM 347)	**0.29 (0.08)**	0.92 (0.41)	0.69 (0.09)	0.73 (0.19)	0.59 (0.04)	1.00 (0)	**0.42 (0.06)**	0.71 (0.06)	0.67 (0.11)	0.77 (0.18)	0.96 (0.39)	0.88 (0.13)	0.94 (0.4)
K. rhizophila (DSM 348)	**0.33 (0.06)**	1.08 (0.31)	0.67 (0.12)	0.92 (0.12)	0.83 (0.12)	1.00 (0)	0.79 (0.16)	1.01 (0.4)	0.96 (0.39)	0.83 (0.12)	0.61 (0.1)	1.00 (0)	1.04 (0.36)
M. luteus (DSM 1790)	**0.23 (0.08)**	0.71 (0.06)	1.02 (0.38)	0.92 (0.41)	0.58 (0.06)	ND	0.88 (0.44)	1.00 (0)	1.04 (0.36)	1.25 (0.35)	0.96 (0.39)	ND	1.04 (0.36)

[a] FIC index values above 2.0 indicate antagonistic effects, values between 0.5 and 2.0 indicate additive effects, and values of less than 0.5 indicate synergistic effects (shown in bold). Values in brackets are standard deviations for three independent measurements.

[b] For organism abbreviations, see Table 1, footnote *b*.

[c] For peptide abbreviations, see Table 1, footnote *a*. ND, not determined.

TABLE 3. HC_{50} values for silver alone or in combination with peptides

Silver and peptide combination	HC_{50} values for indicated agents[a]:							
	$AgNO_3$	AgNP	PGLa	Mag2	GS	PMB	Alam	GA
Silver or peptide alone	11.5 (1.8)	>1,024	165 (11.2)	202.2 (11.5)	10.1 (2.6)	210.7 (30.1)	17.9 (2.4)	2.3 (1)
Peptide + 2 μg/ml $AgNO_3$			64.8 (5.4)	141.4 (11)	1.3 (0.2)	11.1 (1)	4.7 (1)	0.7 (0.1)
Peptide + 8 μg/ml $AgNO_3$			11 (2.6)	34.2 (5.1)	1.2 (0.2)	1 (0.1)	2.2 (0.5)	0.1 (0.01)
Peptide + 8 μg/ml AgNP			120.3 (27.8)	184.8 (12.2)	4.8 (0.8)	56.7 (5.7)	17.6 (2.5)	1.7 (0.3)
Peptide + 32 μg/ml AgNP			63.5 (6.9)	132.8 (20.9)	3.2 (0.9)	34 (4.8)	17.4 (2.6)	1.5 (0.1)

[a] HC_{50} values are in μg/ml. Values in brackets are standard deviations for three independent measurements. For abbreviations of antibacterial agents, see Table 1, footnote *a*.

hemolytic activity even at very high concentrations (1,024 μg/ml).

Our data thus show that silver nanoparticles together with all antimicrobial peptides tested exhibit at least an additive effect. Genuine synergy was observed when silver nitrate and silver nanoparticles were used together with polymyxin B and gramicidin S. The combination of silver nanoparticles and polymyxin B showed the most pronounced antibiotic synergy against gram-negative bacteria. The nanoparticles also exhibited a remarkably low level of hemolytic activity, unlike $AgNO_3$. The combination of silver nanoparticles with polymyxin B is thus a promising candidate for a new treatment for infections caused by gram-negative pathogens. Mechanistically, it appears that permeabilization of the outer bacterial membrane by polymyxin B enhances the intrinsic antibiotic effect of the silver nanoparticles.

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